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# THE IN VITRO CULTURE OF PINUS TAEDA L. CALLUS TISSUE ON A CHEMICALLY DEFINED MEDIUM

A Thesis

by

Samuel A. Alexander, B.S. 1964

SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

in the

GRADUATE SCHOOL

of

LOUISIANA TECH UNIVERSITY

AUGUST 1970

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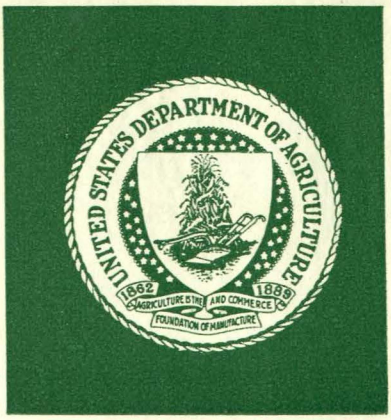


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# THE *IN VITRO* CULTURE OF *PINUS TAEDA* L. CALLUS TISSUE ON A CHEMICALLY DEFINED MEDIUM

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## ABSTRACT

Stem explants of loblolly pine, Pinus taeda L., were cultured in vitro on a modified Harvey's (1967) chemically defined medium containing (g/l): $Ca(NO_3)_2$, 0.5; $MgSO_4$, 0.14; $KH_2PO_4$, 0.3; $(NH_4)_2SO_4$, 0.025; $FeSO_4$, 0.014; $MnSO_4$, 0.0035; glucose, 20.0; 2,4-D, 0.002; and 0.6 percent Bacto agar in double distilled water, pH 5.6. Stem tissue from one month old greenhouse seedlings were surface sterilized two to three minutes in 1/1000 $HgCl_2$ solution, twice washed in sterile distilled water, cut into 5 mm sections, and implanted in the medium. This procedure as well as other methods of surface sterilization proved inadequate for controlling contamination of field collected diseased and normal tissue. Cultures were incubated under constant light, 250 to 350 foot candles, at 18 to 22° C. Primary callus cultures were light green in color with a rough texture and produced enough callus to be subcultured in three to four weeks. Subcultures were grown under similar conditions with a modified Harvey's (1967) vitamin complex added at (mg/l): kinetin 1.0; ascorbic acid, 0.1; nicotinic acid, 0.5; choline Cl, 1.0; Ca pantothenate, 0.1; thiamine HCl, 1.0; pyridoxine HCl, 0.5; urea, 20.0; and myo-inositol, 100.00. Subcultures were light yellow in color with a rough texture and appeared to double in size in about four weeks.




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# INTRODUCTION

Loblolly pine, Pinus taeda L., is the leading commercial timber species in the southern United States and has been referred to as the second most important timber species in the world. The range extends along the Atlantic and Gulf Coastal plains (Harlow and Harrar, 1958). The average harvest of loblolly pine is five billion board feet and with its site tolerance, it can be expanded to an even greater volume. In the South, the wood using industry has developed into a five billion dollar a year enterprise (Wahlenberg, 1960).

Southern fusiform rust, caused by Cronartium fusiforme Hedgc. and Hunt ex Cumm., is a major disease of loblolly pine. Samples of 113,000 trees in five southern states revealed that fusiform rust infections ranged as high as 56 percent in slash and loblolly pine plantations (Peterson and Jewell, 1968). Wakeley (1954) considers C. fusiforme the second most serious southern pine nursery disease.

As defined by Arthur (1934), C. fusiforme is a heteroecious rust fungus requiring spore stages on coniferous and dicotyledonous species. In the South, one of the principal hosts is P. taeda. This rust attacks stems and branches of the southern pine where it is expressed as a spindle-shaped gall, but is limited normally to infecting only the leaves of the angiosperm hosts. The latter are mainly in the genus Quercus (the oaks) with the principal hosts being: Q. nigra L., water oak; Q. phellos L., willow oak; and Q. laurifolia Michx., laurel oak. The rust is most damag-

ing to the coniferous host, causing little or no damage to oaks (Verrall, 1958). The spore stages of C. fusiforme on pine are the pycniospores and and the aeciospores. The function of the former is unknown, but Hirt (1964) reported pycniospores of C. ribicola Fischer, a close relative of C. fusiforme, to have no function. Aeciospores are released in early spring and infect new leaves of the oak. The fungus cannot spread from pine to pine by these spores. Normally, the first spores to be produced on the oak following aeciospore infection are the urediospores. These are followed by the teliospores which in turn germinate in place to produce the typical four-celled basidium. Each cell of the basidium then produces a basidiospore which when released and carried by wind infect the pines either through cotyledons, primary needles, or new succulent stem tissue. A gall is formed on the pine in about nine to ten months (Hedgecock and Siggers, 1949, Verrall, 1958, Jewell, 1960).

Most rust fungi have been considered to be obligate parasites; i.e. they could not be cultured on chemical media in the laboratory. Thus, the study of these fungi had to be under natural conditions or in prepared slides for microscopic examination. This limited greatly the type of research possible with these fungi. Investigators have been desirous for many years of obtaining laboratory cultures of the rusts. This would permit morphological and cytological study to be made of these fungi in the more natural living condition. However, such research must of necessity first involve the tissue culturing of the host plant. In many cases, this has proved to be a problem. The purpose of these experiments was to develop a chemically defined medium suitable

for *in vitro* culturing of callus tissue from *P. taeda* and eventually induce infection of these cultures with the obligate parasite *C. fusiforme*.

## LITERATURE REVIEW

In 1902, Haberlandt, using the concept of the cell theory, assumed that there were no limits of divisibility down to the cell level. He outlined a discipline which crystallized into modern day tissue culture. Defined by Cutter (1959), plant tissue culture is the aseptic, *in vitro* growth of relatively undifferentiated cell masses derived from any plant part. Working with chlorophyll bearing cells, Haberlandt assumed that the need for artificially providing carbohydrates would be eliminated, but was never able to culture plant tissue because green cells are relatively mature, highly differentiated, and meristematic function is low. Between 1910 and 1915, Harrison, Burrows and Carrell developed the techniques and media for animal tissue culture. In 1922, Walter Kotte successfully cultured excised root tips. At about the same time, Robbins obtained similar results using excised root tips. During the 1930's R. J. Gautheret reported on the cultivation of cambial tissue from *Salix* and *Populus*. In 1934, P. R. White demonstrated the possibility of growing excised roots of tomato *in vitro*. In 1939, Gautheret, Nobecourt, and White published descriptions of the successful cultivation *in vitro* of carrot and tobacco cambial tissue for long periods of time (White, 1963).

Trione et al, (1968) studied the *in vitro* culture of somatic wheat callus tissue. Callus production occurred most frequently when

Hildebrandt's "D" medium was supplemented with a chelated iron. Growth on agar was superior to that in shake culture. On Torrey and Reinert's suspension medium, branching and enlargement of the root system resulted but very little proliferation of callus occurred.

Cutter (1960) reported the successful in vitro culture of Arisaema atrosubens (Ait.) Blime, Jack-in-the-pulpit. The callus tissue was infected with Uromyces ari-triphyllum (Schw.) Seeler, an autoecious rust. Blocks of the infected tissue were surface sterilized and placed on Gautheret's agar containing 10 micrograms of either indoleacetic acid (IAA) or naphthaleneacetic acid (NAA). After 10 days in culture, the tissue turned bright green and formed callus. Aecia and aeciospores were produced after three to four months in culture. Later pale yellow mycelial pustules were formed. These coalesced growing down to the agar, but the mycelium had slow growth and never formed spores. A. atrosubens growing under field and greenhouse conditions were inoculated with the pathogen and subsequently became diseased, indicating that the organism growing saprophytically was U. ari-triphyllum. Five strains of the rust were eventually isolated and grown in axenic culture.

A synthetic medium for callus production of Fraxinus pennsylvanica Marsh., green ash, was developed by Wolter and Skoog (1966) on a modified Reinert and White inorganic nutrient solution supplemented with vitamins and an auxin, 2,4-dichlorophenoxyacetic acid (2,4-D). Myo-inositol, pyridoxine and 2,4-D were found to be essential for growth.

Winton (1968) used a modified Wolter's liquid medium to grow callus tissue of aspen, Populus tremuloides Michx. Callus tissue was obtained

with 2,4-D at 0.5 and 0.04 mg per liter. At the high concentration, the tissue grew rapidly and formed tan, rough surfaced spheres of callus. Light yellow, uniformly smooth and firm spheres of callus were produced at the low concentration.

A callus cell suspension of Acer pseudoplatanus L., sycamore maple, was grown by Simpkins and Street (1970) on a synthetic medium with two percent glucose, 1.0 mg per liter of 2,4-D and kinetin at 0.25 and 2.5 mg per liter. It was found that kinetin at 2.5 mg per liter retarded growth while 0.25 mg per liter stimulated growth.

Steinhart et al, (1961) have maintained callus tissue of Norway spruce, Picea abies (L.) Karst., seedlings for three and one-half years. Callus was grown using a medium containing five mineral salts, eight trace elements, a vitamin complex, and malt extract. IAA and NAA stimulated growth whereas 2,4-D with kinetin did not. Myoinositol was the only compound that stimulated callus formation. Cultures were maintained under a constant weak light at 22 to 23° C. Slight changes in pH had no noticeable effect on callus production.

White (1967a and 1967b) studied the in vitro culture and the site of callus formation of white spruce, P. glauca (Moench.) Vess. Normal and tumor cultures were maintained on a medium containing five percent agar, White's mineral salts, vitamins, 2,4-D, a sugar and a source of organic nitrogen. Cultures were grown at 25° C in darkness or under 16 hours of light. To study the site of callus production, discs of white spruce tissue were cultured on Risser and White's standard nutrient medium. Discs contained xylem, phloem and cambial cells. Cores were

collected from 20 to 60 year old trees. Older bark was removed, treated with 95 percent ethyl alcohol, divided into four equal parts and implanted. Callus was initially formed from the cambium or current year's phloem parenchyma, then older phloem parenchyma became active and formed additional cells.

In 1951, Hotson and Cutter (1951) reported the culture of a rust fungus on artificial media in the absence of its host. The cedar-apple rust, Gymnosporangium juniperi-virginianae Schw., forms telia galls on its coniferous host Juniperus virginiana L., eastern red cedar and the aecial stage on species of Crataegus. Telial galls were soaked for two minutes in 95 percent ethyl alcohol, then the outer cortex aseptically removed, and the inner parenchyma tissue sectioned into small cubes. The cubes were dipped in 0.1 percent ascorbic acid then placed on Gautheret's nutrient solution Number 4, containing three percent dextrose and 500 ppm of ascorbic acid. Three to four months were required for the cultures to double in weight, after which time some original tissues produced typical telial horns with viable teliospores. According to these investigators, a white fluffy aerial mycelium formed at the base of some telial horns, but when transferred to fresh medium it did not grow. Bits of mycelium, from one infected callus culture, were used to inoculate 12 uninfected callus cultures of Crataegus. Three months later, three of these inoculated cultures developed typical roestelia of Gymnosporangium in which aeciospores and peridial cells were produced in a normal fashion. Cutter (1959) reported the isolation of seven strains of G. juniperi-virginianae and subsequent growth of all strains in axenic culture.

In an attempt to duplicate Hotson and Cutter's work with Gymnosporangium, Mahsehwai et al, (1967) used callus from infected material and uredospores to artificially inoculate healthy callus. Both techniques failed to infect healthy, proliferating callus. It was suggested that rapid growth of the host may cause the callus to remain sterile.

Barnes and Naylor (1959) established root callus cultures of five southern pines: pond pine (P. serotina Michx.), longleaf pine (P. palustris Mill.), slash pine (P. elliottii Englm.), sand pine (P. clausa (Champ.) Vasey), loblolly pine (P. taeda L.). Callus tissues were maintained on Heller's mineral medium supplemented with sucrose, tyrosine, biotin, pantothenic acid and IAA. Copper was deleted. Tissues were sterilized by washing with 70 percent ethyl alcohol for one to two minutes followed by flaming for 30 seconds. Each tissue section was half submerged in the agar medium, then incubated at 18 to 20° C in darkness. Glycine, at 3 ppm, inhibited callus formation in all species tested, and thiamine, above 1 ppm, inhibited callus production in sand pine. Pyridoxine was found to be essential for growth regardless of species.

Tissue of western white pine, P. monticola Dougl., has been cultured in vitro on chemically defined media by Harvey (1967). Stem sections from the current year's shoots of three to seven year old seedlings were rinsed in rapidly running water for five hours, then sterilized for 20 minutes in 5.25 percent sodium hypochlorite. Bark was aseptically removed and sections were implanted on a medium containing 15 ml of two percent Difco malt extract and 0.8 percent Noble agar. Cultures were incubated for two weeks at 20° C under

400 foot candles of light, then transplanted to various media and incubated for four weeks. Sections on media containing kinetin and 2,4-D grew at a faster rate than sections on media containing IAA and NAA. Cultures with 2,4-D showed no tendency to lose vigor. Glucose and sucrose were equivalent as carbon sources. Urea and tyrosine enhanced growth. Ascorbic acid was added to control enzymatic browning.

Utilizing the same procedures and nutrient medium, Harvey and Grasham (1969a) cultured callus tissue of Norway spruce (P. abies), blue spruce (P. pungens Englem.), Engelmann spruce (P. engelmannii Engelm.), whitebark pine (P. albicaulis Engelm.), lodgepole pine (P. contorta Lous.), limber pine (P. flexilis James), Austrian pine (P. nigra Arn.), ponderosa pine (P. ponderosa Laws.), western red cedar (Thuja plicata D. Don.), Douglas fir (Pseudotsuga menziesii (mirb.) Franco), lowland white fir (Abies grandis Lind.), and western larch (Larix occidentalis Nutt.). Several vitamins and amino acids increased growth, and all species demonstrated an absolute requirement for an exogenous supply of auxin. However, the level and type of auxin varied widely with the different species.

Brown and Lawrence (1968) have cultured callus tissue of longleaf pine, P. palustris, on a modified synthetic tobacco medium. Cultures were maintained for 18 months through 12 transfers. Hypocotyls of 30 day old seedlings were cut into sections, split longitudinally, and aseptically placed on the medium. Subcultures were made every three to four weeks. They also reported that loblolly and slash pine tissues grew slightly on the same medium.

Walkinshaw et al, (1965) have reported the *in vitro* culture of slash pine, *P. elliottii*, infected with *C. fusiforme*. Stems of infected three to nine month old seedlings were sectioned and placed in 70 percent ethyl alcohol for two to three minutes then flamed for 30 seconds. Sections were placed on the medium of Barnes and Naylor which contained 0.1 percent oleic acid, 0.3 percent sucrose and 0.8 percent agar. Tyrosine was deleted. Sections were half submerged, then incubated at 18 to 20° C in darkness. Slash pine tissue cultures were maintained through three to four transfers. Healthy callus was maintained for three months while infected cultures were maintained through the fifth month. Throughout this period typical rust haustoria were observed in primary tissue only.

In 1968, Koenigs reported the culturing of *C. ribicola* in callus of western white pine. Healthy and infected two to three year old seedlings were sectioned, surfaced sterilized, rinsed in sterile distilled water, then placed on a medium containing two percent Difco malt extract and two percent Difco Bacto agar. Cultures were incubated at 20° C under 200 to 250 foot candles of light for two to four months. A ring of callus developed over the top of stem sections after two to three weeks. *C. ribicola* haustoria were observed in 90 percent of the explants known to have contained rust mycelium. Following the establishment of the fungus, the mycelium grew throughout new tissue. The host tissue was green. Contamination ranged from 18 to 100 percent in different experiments, however removal of the periderm reduced the occurrence of contamination. Cultures which did not survive, discolored after one month.

Harvey and Grasham (1969b) reported the culturing of C. ribicola in callus of western white pine using a similar procedure and medium for the growth of western white pine callus. Ninety percent of the infected primary explants, grown under standard conditions of 16 hours of light at 70° F and eight hours of darkness at 40° F, survived and produced callus tissue. Aerial hyphae were present in 55 percent of the surviving cultures after six weeks and completely covered the callus after 12 weeks. Aerial hyphae were produced throughout a pH range of 4.0 to 8.0. Sporulation occurred only on tissue taken from the margins of two year old galls.

Harvey and Grasham (1969c) also reported that tissue cultures from stem cortex and leaf tissue of western white pine have been artificially inoculated with mycelium of C. ribicola. Healthy stem cortex tissue cultures were obtained using the technique and medium of Harvey, 1967. Current year needles were surface sterilized in 5.25 percent sodium hypochlorite for one minute, then rinsed several times in sterile distilled water. Needles were cut into 5 mm sections and placed on the same medium. Inoculations occurred either by transferring the rust mycelium to healthy tissue or by placing uninfected tissue next to infected tissue forcing the rust mycelium to grow across the intervening space. Regardless of method, establishment of rust infection occurred and was well established in two to six weeks. Infection was completed in a shorter period of time when needle tissue was used as compared to stem cortex tissue.

Harvey and Grasham (1970) have cultured C. ribicola in the absence of any physical contact with its host. Healthy host tissue

cultures were grown for two months, then covered to a depth of 2.5 cm with the culture medium. The surface of the medium was covered with a cellophane membrane previously boiled in distilled water for one hour. Two to three ml of Noble agar were deposited on the membrane to provide a moist surface for the inoculum. Pieces of mycelium and heavily infected tissue were placed on the Noble agar. Cultures were incubated for 16 hours at 70° F and 400 foot candles of light, and eight hours at 40° F and no light. After 90 days, all cultures had produced vegetative growth on Noble agar. Identity of the fungus was made by reinfection of host tissue and observation of typical rust mycelium.

## MATERIALS AND METHODS

Loblolly pine, P. taeda, was used exclusively as the source of experimental plant material. Stem and needle sections were obtained from one year old nursery seedlings, six to eight week old seedlings grown aseptically in vitro, four to six week old seedlings grown in pots under greenhouse conditions, or young shoots from five to fifteen year old trees. Diseased material was naturally infected with C. fusiforme. Cultures were maintained at 18 to 22° C and 250 to 350 foot candles of light from 20 watt, cool white fluorescent lamps, except where stated differently.

Media were prepared using double distilled water. Single distillation was accomplished with a Stokes gas water still (Pennsalt Chemicals Corporation, Philadelphia, Pennsylvania) and redistillation with a Barnstead electric redistiller (Barnstead Sybron Corporation, Boston, Massachusetts). Glassware was washed first in 6 N HCl for one hour followed by two washings in distilled water. Stender culture dishes, 63 by 29 mm (Variety Glass Incorporated, Cambridge, Ohio), were sterilized in a hot air oven for three hours at 160° C. Transfers were carried out in a transfer hood. A Sherer growth chamber (Sherer-Gillett Company, Marshall, Michigan) with a capacity of 80 culture dishes and a growth chamber constructed locally with a capacity of 40 culture dishes were used. Screw cap tubes, containing 25 ml of media, were autoclaved for 15 minutes at 121° C and 15 psi. Following sterilization

the contents of one tube was used to fill one culture dish. Jewell's (1962) microtechnique procedure was followed preparing slides of the plant material except safranin replaced orseillin BB as the primary stain for slides one through 40.

## Experimental Test I

One year old loblolly pine seedlings, diseased and normal, were collected at the Continental Can Corporation Nursery, Hodge, Louisiana. Loose bark and needles were removed and the seedlings washed in running water for one hour. Tops and roots were discarded and stems rinsed for 10 minutes in distilled water. Following the second rinse, bark was removed with single edge razor blades which had been sterilized with 70 percent ethyl alcohol for approximately one minute, removed and flamed for 15 seconds as recommended by Walkinshaw et al, 1965. Flamed sections were transferred to culture dishes and placed in a growth chamber under 14 hours light and 10 hours darkness. A total of 69 cultures were prepared. The pH was not adjusted. The culture medium used is listed in Table 1.

## Experimental Test II

Experimental test II was similar to test I except for the decontamination procedure. Stems free of loose bark, needles, tops and roots were washed in tap water for one hour, then placed in six percent sodium hypochlorite for five minutes. Remaining bark was removed and the stems cut into 5 mm segments, bathed in 70 percent ethyl alcohol for one minute, then flamed for 15 seconds. After flaming, each section was placed separately in a test tube containing 10 ml of sterile distilled water then transferred to the culture dishes. The culture medium used is listed in Table 1.

## Experimental Test III

Decontamination was similar to test II, except bark was not removed. Diseased and normal stems were collected from previous year's growth of five to 15 year old trees and cut into 5 $mm^3$ sections. Three types of media were employed and are listed in Table 2. The pH of media B and C was adjusted to 5.6 using 0.1 N KOH, and medium A had an unadjusted pH of 5.7. Cultures were maintained in a growth chamber under 14 hours light and 10 hours darkness.

## Experimental Test IV

Media were similar to that in test III. Diseased and normal tissue from six month old stems were collected. After loose bark and needles were removed, the stems were washed in running water for two hours, then placed in six percent sodium hypochlorite for 10 minutes, and twice washed in sterile distilled water with each washing lasting 10 minutes. Tissue sections of approximately 5 $mm^3$ were excised and placed on culture medium. Cultures were maintained in a growth chamber at 14 hours light and 10 hours darkness.

## Experimental Test V

Diseased and healthy tissue were collected and washed for four hours after needles and loose bark had been removed. Specimens were divided into two groups and surface sterilized with six percent sodium hypochlorite. Group 1 was sterilized for 45 minutes and group 2 for 30 minutes. Following sterilization, sections were washed in sterile distilled water twice for 30 minutes per washing. Tissues were cut into 0.5 to 1.5 cm sections and immediately implanted on culture media, then transferred to a growth chamber. The pH of media B and C was adjusted to 5.6 and 5.5 respectively using 0.1 N KOH.

The pH of medium A was an unadjusted 5.7. The three media used in test V are listed in Table 3.

Experimental Test VI

The surface sterilization procedure for test VI was similar to that employed in test V. However, in the former, tissues submerged in sodium hypochlorite were exposed to a vacuum. After removal from the vacuum chamber, specimens were washed, cut into sections, implanted and transferred to a growth chamber. The media used in test VI are shown in Table 3 except that NaCl at 200.0 mg was added and $FeSO_4$ replaced $Fe_2(SO_4)_3$ in medium B, and oleic acid was deleted from medium C.

Experimental Test VII

Stratified seeds (supplied by Continental Can Company) were surface sterilized in 1/1000 $HgCl_2$ solution for two minutes, rinsed in sterile distilled water twice at 15 minutes per washing, then implanted in culture tubes containing 10 ml of the growth medium. Seeds were allowed to germinate in a growth chamber. Stem and needle sections from four to six week old seedlings, growing aseptically in vitro, were transferred to culture dishes, then placed in a growth chamber. The growth medium used in test VII is listed in Table 4. The pH of the medium was adjusted to 5.6 with 0.1 N KOH.

Experimental Test VIII

Test VIII was the same as test VII except 1.0 mg kinetin was added to the medium.

Experimental Test IX

Stratified seeds were allowed to germinate in a moist chamber. The germinating seeds were placed in 10 inch pots containing sterile sand and allowed to grow under greenhouse conditions. One month old

seedlings were removed and sand particles washed from the root systems. Roots and tops were excised and discarded. Stems were surface sterilized in a 1/1000 $HgCl_2$ solution for two to three minutes, then rinsed in sterile distilled water three times at 15 minutes per washing. The material was cut into 5 mm sections, transferred to the culture media, then placed in a growth chamber for 30 days. The pH of the media was adjusted to 5.6 using 0.1 N KOH. A liter of each medium was divided into four 250 ml portions. To three portions, 0.1, 1.0 and 2.0 mg of 2,4-D were added respectively. 2,4-D was not added to the last portion. The media used in test IX are listed in Table 5.

## Experimental Test X

Experimental procedure for test X was similar to test IX except the plant material was current year shoots from 10 to 15 year old trees.

## RESULTS

As shown in Table 6, experimental tests I, IV, V, VI and X were terminated from four to 15 days after initiation due to contamination. Test II, after 63 days in culture, had 20 percent contamination. The uncontaminated cultures did not turn brown and appeared to be living; however, no callus was produced. Fifteen days after initiation of test III, all cultures turned brown and were assumed to be dead. Contamination appeared in 21 percent of the cultures and no callus was produced.

The duration of test VII was 38 days with 20 percent contamination. Seven primary cultures produced callus, three from needles and four from stems. The callus tissue was light green in color with a smooth texture. Four of the primary callus tissues, two needles and two stems, were subcultured to fresh medium after 30 days in culture. One stem callus subculture immediately proliferated but turned brown after the eighth day. Contamination occurred in 20 percent of the cultures in test VIII with nine cultures producing callus. The callus tissue was smooth and light green in color. All cultures turned brown after 42 days in culture.

In experimental test IX, 16 primary cultures produced callus. Of these, all but one were produced on media A and B containing 1.0 and 2.0 mg 2,4-D respectively, as shown in Table 7 and Figure 1. Using medium A, 20 and 40 percent callus were produced with 1.0 and

2.0 mg 2,4-D respectively, and on medium B, 40 and 50 percent callus were produced with 1.0 and 2.0 mg 2,4-D respectively. One callus culture was produced on medium C which contained 2.0 mg 2,4-D but the primary culture produced only 10 percent as much callus as the other primary cultures. Two of the primary callus cultures were subcultured and all others sacrificed for slide preparation. Microscopic examination revealed callus formation and active cell division as shown in Figures 2 and 3. Primary callus cultures on medium B began to turn brown after 30 days in culture, while those on A did not turn brown after 60 days in culture. One primary callus culture, after initial callus removal, produced new callus tissue. The subcultures were transferred to medium C without glycine. The subcultures continued to proliferate for 30 days and then began to turn brown. On the 31st day, these tissues were transferred to fresh medium C without glycine. Several of the primary cultures on media containing 2.0 mg 2,4-D ruptured from rapid cell division, as shown in Figure 4. The primary and secondary callus tissues were light greenish yellow in color with a smooth to rough texture. Figure 8 depicts a primary stem callus section cultured on media containing 2,4-D at 2.0 mg, six mineral salts, and glucose.

Table 1. Composition of culture medium used in experimental tests I and II.

| Chemicals and Amounts per Liter of Aqueous Solution[a] | | | |
|---|---|---|---|
| $KNO_3$ | 717.1 mg | Biotin | 0.1 |
| $KH_2PO_4$ | 394.4 | p-aminobenzoic acid | 0.1 |
| NaCl | 472.0 | Ca-pantothenate | 0.1 |
| $MgSO_4$ | 123.6 | Ascorbic acid | 0.1 |
| $CaCl_2$ | 57.1 | Choline Cl | 1.0 |
| $NH_4Cl$ | 134.5 | Nicotinic acid | 0.5 |
| $FeSO_4$ | 14.0 | Pyridoxine HCl | 0.5 |
| $MnSO_4$ | 3.5 | Thiamine HCl | 1.0 |
| $H_3BO_3$ | 0.18 | Kinetin | 1.0 |
| $ZnSO_4$ | 0.023 | Arginine | 20.0 |
| $NiSO_4$ | 0.0075 | Urea | 20.0 |
| $AlCl_3$ | 0.0075 | 2,4-D | 0.1 |
| KI | 0.0075 | Oleic acid | 1 ml |
| Riboflavin | 0.1 | Glucose | 30.0 g |
| Myo-inositol | 100.0 | Bacto agar | 8.0 |
| Folic acid | 1.0 | | |

[a] Heller's mineral solution (Gautheret, 1955) and Harvey's vitamin complex (1967).

Table 2. Composition of culture media used in experimental tests III and IV.

| Medium | Chemicals and Amounts per Liter of Aqueous Solution | | | |
|---|---|---|---|---|
| A[a] | Malt Extract | 20.0 g | | |
| | Bacto Agar | 20.0 | | |
| B[b] | $Ca(NO_3)_2$ | 500.0 mg | $MnSO_4$ | 3.5 |
| | $MgSO_4$ | 140.0 | 2,4-D | 0.1 |
| | $KH_2PO_4$ | 140.0 | Glucose | 50.0 g |
| | $(NH_4)_2SO_4$ | 25.0 | Noble agar | 8.0 |
| | $Fe_2(SO_4)_3$ | 14.0 | | |
| C[b] | $Ca(NO_3)_2$ | 500.0 mg | 2,4-D | 1.0 |
| | $MgSO_4$ | 140.0 | Kinetin | 1.0 |
| | $KH_2PO_4$ | 140.0 | Ascorbic acid | 0.1 |
| | $(NH_4)_2SO_4$ | 25.0 | Nicotinic acid | 0.5 |
| | $Fe_2(SO_4)_3$ | 14.0 | Choline Cl | 1.0 |
| | $MnSO_4$ | 3.5 | Ca pantothenate | 0.1 |
| | Inositol | 100.0 | Pyridoxine HCl | 0.5 |
| | Tyrosine | 40.0 | Thiamine HCl | 1.0 |
| | Urea | 20.0 | Glucose | 20.0 g |
| | Arginine | 20.0 | Noble agar | 8.0 |

[a]Koenigs (1968)
[b]Harvey (1967)

Table 3. Composition of culture media used in experimental tests V and VI.

| Medium | Chemicals and Amounts per Liter Aqueous Solution | | | |
|---|---|---|---|---|
| A[a] | Malt Extract | 20.0 g | | |
| | Bacto Agar | 10.0 | | |
| B[b] | $Ca(NO_3)_2$ | 500.0 mg | Biotin | 0.1 |
| | $MgSO_4$ | 140.0 | Ca pantothenate | 0.1 |
| | $KH_2PO_4$ | 300.0 | IAA | 1.0 |
| | $(NH_4)_2SO_4$ | 25.0 | Oleic acid | 1 ml |
| | $Fe_2(SO_4)_3$ | 14.0 | Sucrose | 30.0 g |
| | $MnSO_4$ | 3.5 | Bacto agar | 8.0 |
| | Thiamine | 1.0 | | |
| C[c] | $Ca(NO_3)_2$ | 500.0 mg | Kinetin | 1.0 |
| | $MgSO_4$ | 140.0 | Ascorbic acid | 0.1 |
| | $KH_2PO_4$ | 140.0 | Nicotinic acid | 0.5 |
| | $(NH_4)_2SO_4$ | 25.0 | Choline Cl | 1.0 |
| | $FeSO_4$ | 14.0 | Pyridoxine | 0.5 |
| | $MnSO_4$ | 3.5 | Thiamine | 1.0 |
| | Inositol | 100.0 | 2,4-D | 1.0 |
| | Tyrosine | 40.0 | Ca pantothenate | 0.1 |
| | Urea | 20.0 | Glucose | 20.0 g |
| | Arginine | 20.0 | Bacto agar | 8.0 |

[a] Modified Koenigs (1968)
[b] Modified Harvey (1967) and Walkinshaw et al (1965)
[c] Harvey (1967)

Table 4. Composition of growth medium used in experimental tests VII and VIII.

| Chemicals and Amounts per Liter of Aqueous Solution[a] | | | |
|---|---|---|---|
| $Ca(NO_3)_2$ | 500.0 mg | Nicotinic acid | 0.5 |
| $KH_2PO_4$ | 300.0 | Choline Cl | 1.0 |
| $MgSO_4$ | 140.0 | Ca pantothenate | 0.1 |
| $(NH_4)_2SO_4$ | 25.0 | Thiamine | 1.0 |
| $FeSO_4$ | 14.0 | Pyridoxine | 0.5 |
| $MnSO_4$ | 3.5 | 2,4-D | 0.1 |
| Urea | 20.0 | Glucose | 20.0 g |
| Glycine | 3.0 | Bacto agar | 6.0 |
| Ascorbic acid | 0.1 | | |

[a] Harvey's (1967) medium with 300.0 mg $KH_2PO_4$ and glycine.

Table 5. Composition of culture media used in experimental tests IX and X.

| Medium | Chemicals and Amounts per Liter of Aqueous Solution[a] | | | |
|---|---|---|---|---|
| A | $Ca(NO_3)_2$ | 500.0 mg | $FeSO_4$ | 14.0 |
| | $MgSO_4$ | 140.0 | $MnSO_4$ | 3.5 |
| | $KH_2PO_4$ | 300.0 | Glucose | 20.0 g |
| | $(NH_4)_2SO_4$ | 25.0 | Bacto agar | 6.0 |
| B | $Ca(NO_3)_2$ | 500.0 mg | $MnSO_4$ | 3.5 |
| | $MgSO_4$ | 140.0 | Kinetin | 1.0 |
| | $KH_2PO_4$ | 300.0 | Glucose | 20.0 |
| | $(NH_4)_2SO_4$ | 25.0 | Bacto agar | 6.0 |
| | $FeSO_4$ | 14.0 | | |
| C | $Ca(NO_3)_2$ | 500.0 mg | Choline Cl | 1.0 |
| | $MgSO_4$ | 140.0 | Ca pantothenate | 0.1 |
| | $KH_2PO_4$ | 300.0 | Thiamine | 1.0 |
| | $(NH_4)_2SO_4$ | 25.0 | Pyridoxine | 0.5 |
| | $FeSO_4$ | 14.0 | Urea | 20.0 |
| | $MnSo_4$ | 3.5 | Inositol | 100.0 |
| | Kinetin | 1.0 | Glycine | 3.0 |
| | Ascorbic acid | 0.1 | Glucose | 20.0 g |
| | Nicotinic acid | 0.5 | Bacto agar | 6.0 |

[a] Harvey's (1967) medium with 300.0 mg $KH_2PO_4$ and 3.0 mg glycine in medium C.

Table 6. Summary of experimental tests results.

| Test | Duration in Days | Tissue Type | Number of Cultures | Number of Callus | Percent Contamination |
|---|---|---|---|---|---|
| I | 4 | W[a] | 69 | 0 | 100 |
| II | 63 | W | 60 | 0 | 20 |
| III | 15 | W | 90 | 0 | 21 |
| IV | 15 | W | 63 | 0 | 98 |
| V | 8 | W | 156 | 0 | 91 |
| VI | 9 | W | 140 | 0 | 100 |
| VII | 38 | TG[b] | 40 | 7 | 20 |
| VIII | 42 | TG | 76 | 9 | 20 |
| IX | 30 | GG[c] | 120 | 16 | 19 |
| X | 5 | W | 120 | 0 | 98 |

[a] Wild grown material
[b] Tube grown material
[c] Greenhouse grown material

Table 7. Percent callus produced in experimental test IX.

| 2,4-D mg/l | Medium A | Medium B | Medium C | Total |
|---|---|---|---|---|
| None | 0 | 0 | 0 | 0 |
| 0.1 | 0 | 0 | 0 | 0 |
| 1.0 | 20 | 40 | 0 | 20 |
| 2.0 | 40 | 50 | 10 | 33 |
| Total Percent | 15 | 22 | 03 | 19 |

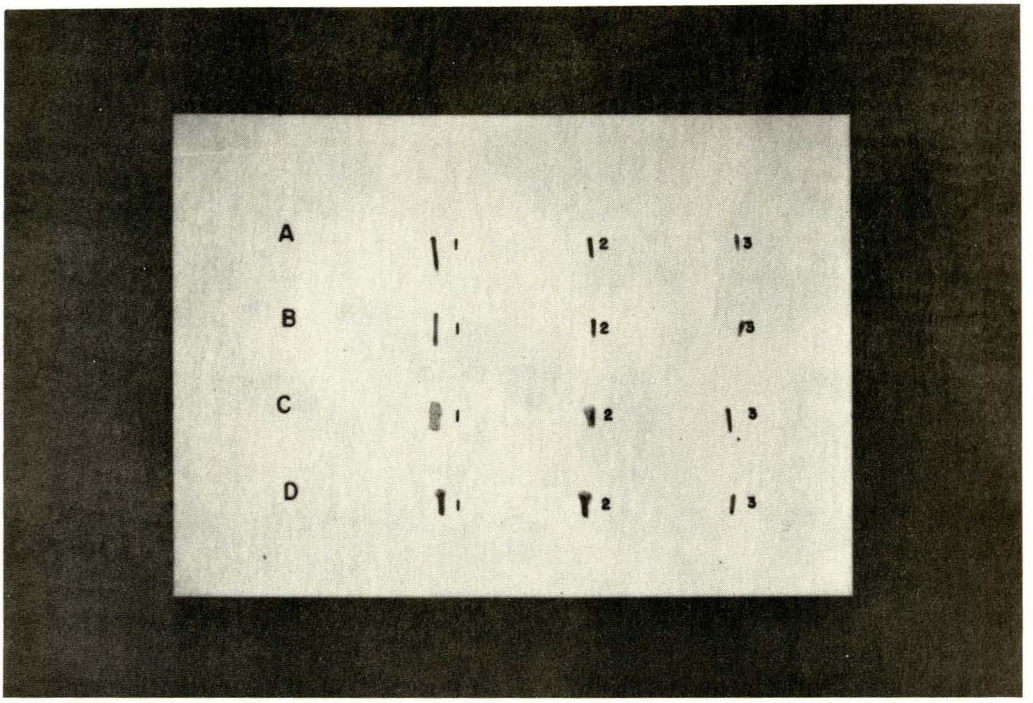


Figure 1. A representative sample of tissue from test IX showing the effect of 2,4-D in three types of media on 30 day old primary stem cultures. A, B, C and D represent 2,4-D at none, 0.1, 1.0 and 2.0 mg/l respectively as shown in Tables 5 and 7. The numbers 1, 2 and 3 are types of media.

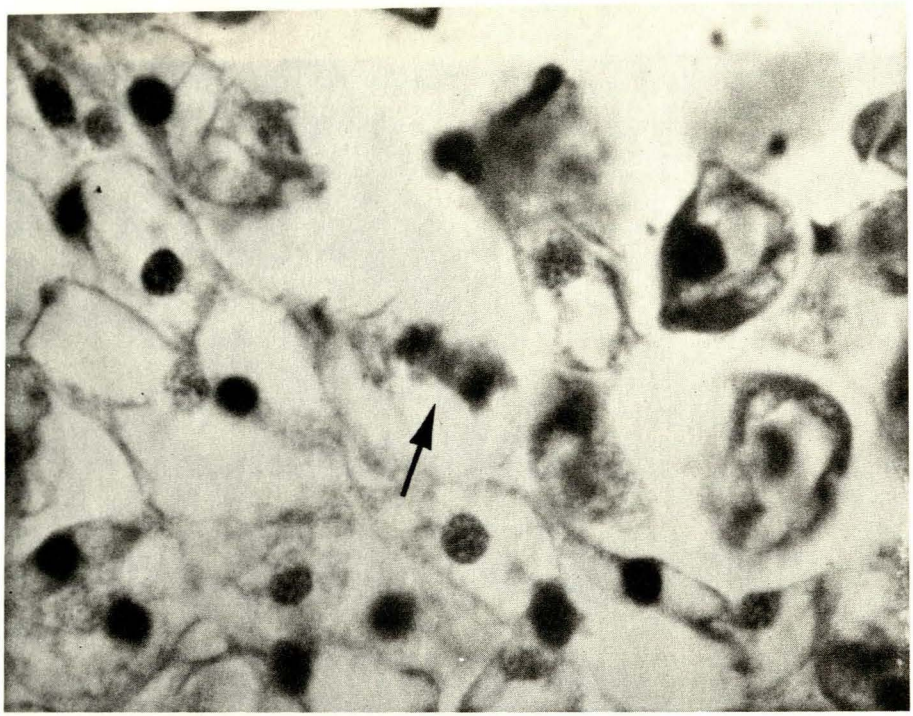

Figure 2. Primary stem callus cells containing one cell (arrow) at the anaphase stage of cell division. X 600

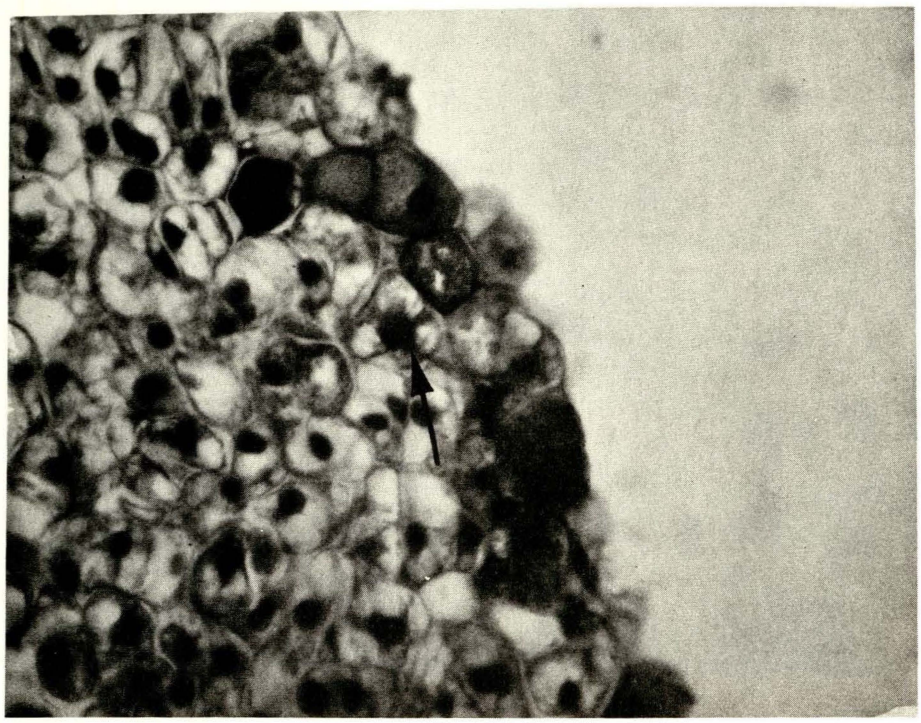

Figure 3. Primary stem callus cells containing one cell (arrow) at early telophase stage of cell division. X 400

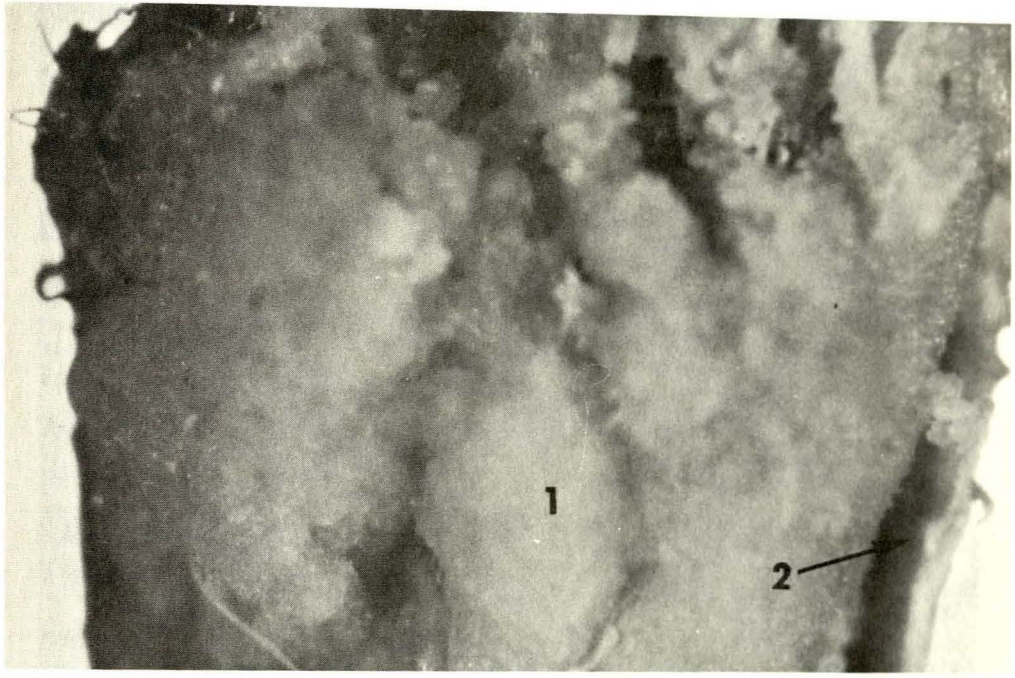


Figure 4. A primary stem callus section, cultured on medium A, Table 5, with 2.0 mg/l 2,4-D. Note that the callus tissue (1) has ruptured the primary tissue (2) due to rapid cell division. X 200

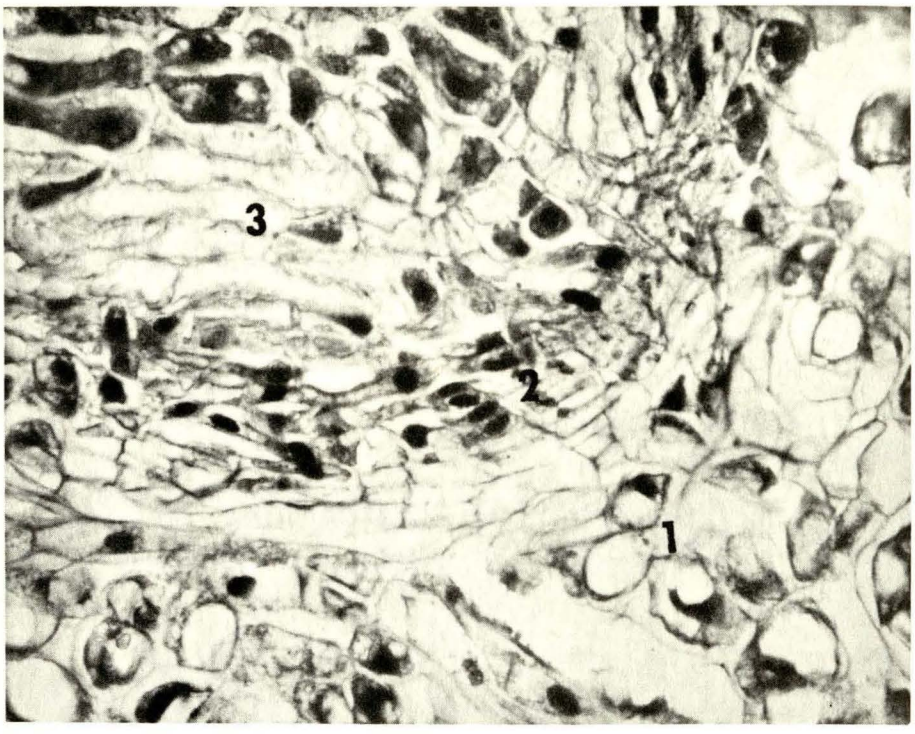


Figure 5. A primary stem callus section showing callus cells (1) originating from periderm (2). The periderm has formed at the end of the xylem tissue (3), a typical reaction to wounding. X 400

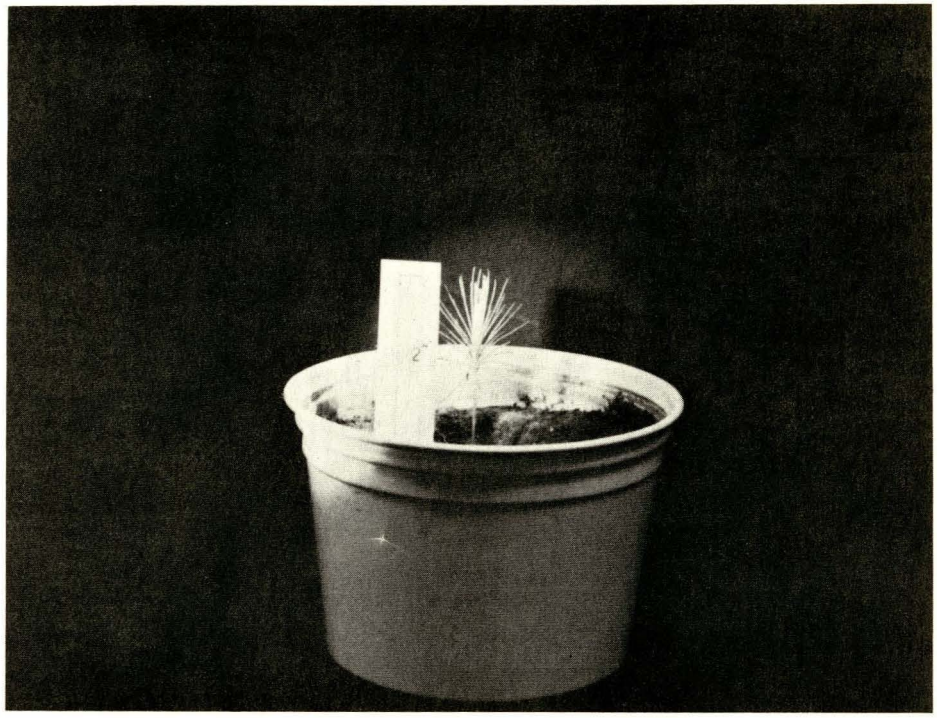

Figure 6. A four week old loblolly pine seedling growing in sterile sand under greenhouse conditions.

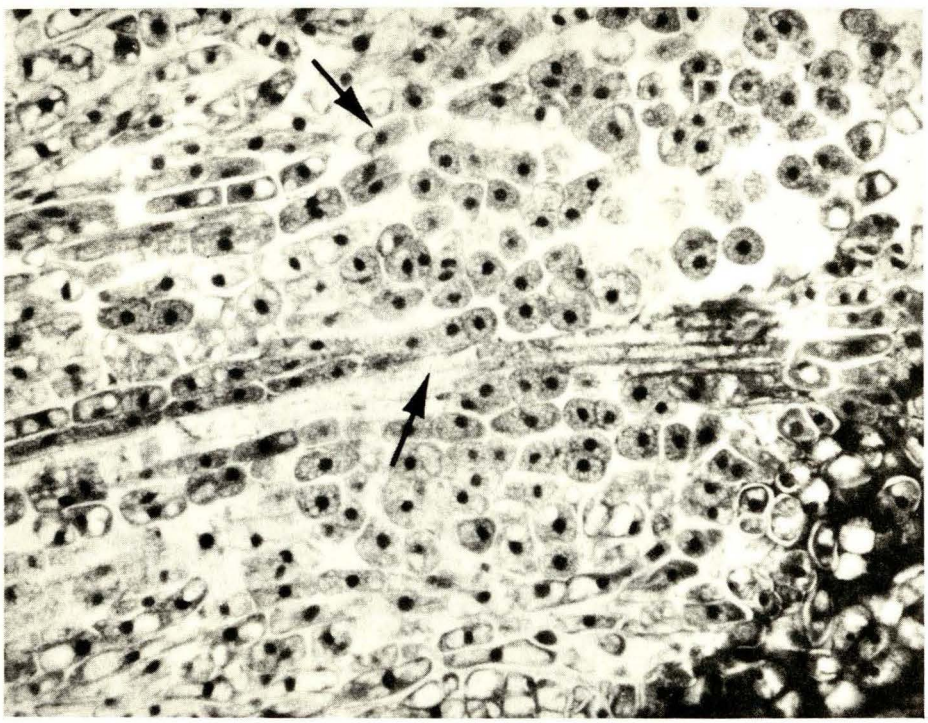

Figure 7. A primary stem callus section depicting the formation of callus cells from the protoxylem (arrow). This section was cultured on a medium containing 2,4-D at 2.0 mg/1. X 400

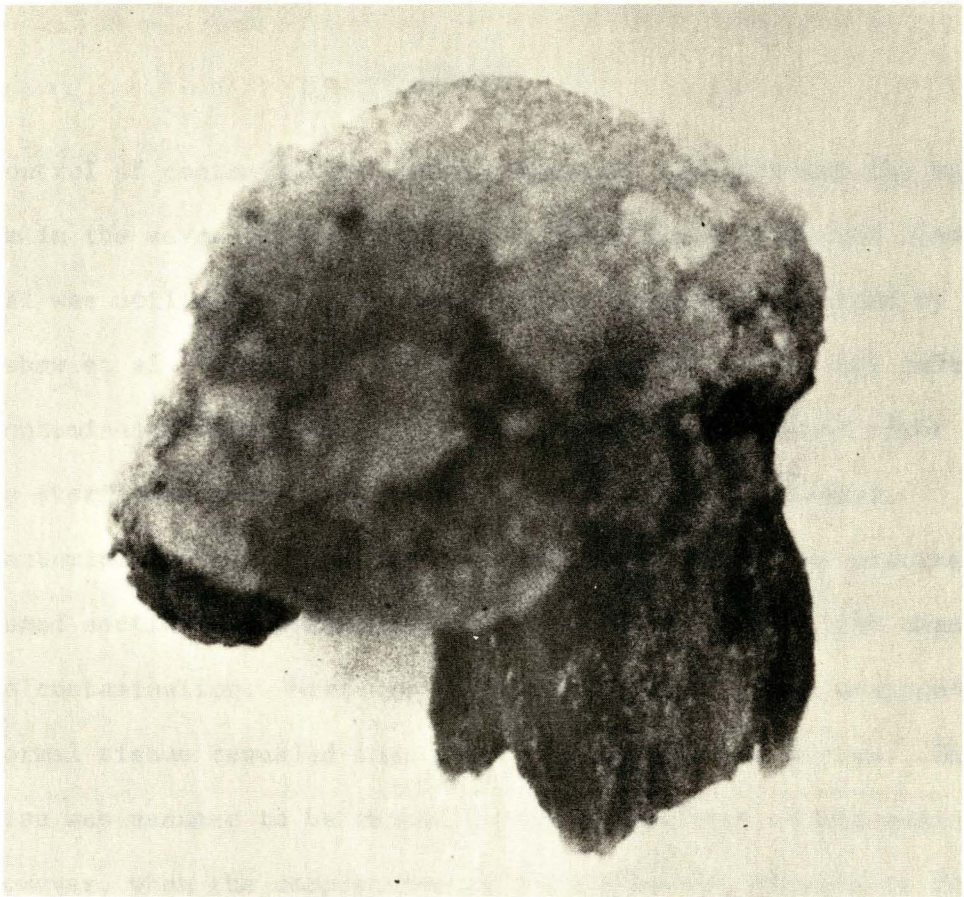

Figure 8. A primary stem callus section which was cultured on a medium containing 2,4-D at 2.0 mg, six mineral salts, and glucose. This callus tissue is three weeks old and the primary stem tissue is producing callus cells for the second time.

# DISCUSSION

Control of contamination without damaging explants was the major problem in the seven experimental tests where field collected plant material was utilized. The decontamination procedure reported by Walkinshaw et al, (1965) was utilized in the first test. All cultures were contaminated within four days. Contamination occurred after surface sterilized sections were placed in a common container.

Contamination was reduced to 20 percent in test II by placing the flamed sections in individual tubes, thus eliminating the chance of mass contamination. Microscopic examination of slides prepared from normal tissue revealed that living cells were not active. This condition was assumed to be caused by the composition of the medium.

However, when the composition of the medium was changed in the subsequent test, all cultures produced a brown color and died within a period of 15 days. Several investigators (Harvey, 1967, and Koenigs, 1968) have reported the problem of enzymatic browning in comparable investigations and have recommended the addition of ascorbic acid as a control measure. Since ascorbic acid was present in this medium, the browning may have been caused by harsh treatment of sections during flaming or by scalpel burning during excision.

The decontamination procedure recommended by Koenigs (1968) was utilized in the fourth test. The scalpel was cooled by dipping in sterile distilled water immediately after flaming. However, this

procedure did not retard contamination. Likewise, contamination control was not achieved by leaving the tissue in the germicide for longer periods of time or placing the tissues in a vacuum with the germicide, as was done in the fifth and sixth tests respectively.

In all of these tests using field collected plant material, contamination was not controlled and callus was not produced. Harvey (1967) and Koenigs (1968) reported successful control of contamination when using field collected plant material. However, the procedures used by these investigators were not effective with field collected material in the present study. This might have been due to environmental conditions favoring the development of certain contaminating organisms. One such organism which occurred in all diseased tissue, was isolated and identified as Pestalotia spp., a weak parasite of loblolly pine.

To alleviate the problem of contamination, plant material was grown aseptically and utilized in test VII. However, 20 percent contamination was noted in this case. Three horizontal needles and four vertical stem cultures produced callus. Callus was light green in color with a smooth texture. Slides were prepared of one callus culture and microscopic examination revealed the tissue originating from a periderm formed at the end of the xylem tissue as shown in Figure 5. This is a typical reaction of normal pine tissue to wounding. After 30 days, two needle and two stem callus cultures were subcultured. All cultures died when the incubation temperature accidentally rose to 40° C.

In test VIII, the contamination was 20 percent with nine stem cultures producing callus. However, these survived only seven days with no successful subcultures developed.

Seedlings grown in sterile sand under greenhouse conditions, as shown in Figure 6, were used in test IX because tube grown seedlings required from six to eight weeks to reach sectioning size. Only 19 percent of the cultures were contaminated. Harvey (1967) used 0.1 mg 2,4-D for growing white pine callus; however, this concentration was found to be inadequate for loblolly pine callus formation. Stem sections implanted in media containing 1.0 and 2.0 mg 2,4-D hypertrophied after 10 to 14 days with callus development occurring a few days later. A microscopic examination of callus tissue revealed many cells at various stages of division as shown in Figures 2 and 3. Callus cells formed from protoxylem tissue which had retained meristematic activity is shown in Figure 7. Periderm was not formed in these callus cultures as compared to test VII. This was possibly due to the higher concentration of 2,4-D in the media which may have stimulated hyperplasia at such a rapid rate that periderm development was totally restricted. Due to hyperplasia, some sections on media containing 2.0 mg 2,4-D split, with callus being formed along the entire length of the section as shown in Figure 4.

Kinetin was not necessary for callus formation on primary cultures although increased growth was noted as indicated in Table 7. This may be due to the presence of the essential growth factors in the primary tissue which caused a reduction in callus formation after 30 days. Futhermore, subcultures would not grow on media without these

growth factors. Primary cultures with kinetin in the media turned brown after 30 days, which may have been due to the high metabolic activity causing depletion of the media. Cultures without kinetin did not turn brown after 60 days.

White (1943) recommended using glycine instead of an amino acid complex; however, according to Barnes and Naylor (1959) glycine inhibited growth of pine tissue. Since only one culture produced callus on media containing glycine and subcultures did not grow on media with glycine although requiring the same media without glycine, this test substantiated the report of Barnes and Naylor. The presence of glycine in the media might explain the erratic results in tests VII and VIII.

The callus tissue formed with 1.0 and 2.0 mg 2,4-D in the media were rough textured with primary callus light green and subcultures light yellow in color. The largest subculture exhibited a brown color after 30 days. It was divided into two sections and each was subcultured. These continued to proliferate for four weeks.

The decontamination procedure of tests VII, VIII and IX was not effective in test X. Since contamination occurred in only five days, this does not preclude the use of the media for callus production of field collected material.

In conclusion, four week old seedlings grown in sand culture, decontaminated with $HgCl_2$, cut into 5 to 10 mm sections, and implanted in Harvey's (1967) mineral salt solution with 2.0 mg 2,4-D, 20.0 g glucose, 1.0 mg kinetin and 6.0 g Bacto agar was a satisfactory technique for the production of primary loblolly pine callus tissues. For subcultures, Harvey's (1967) vitamin complex must be added to the

mineral salt medium. This medium should also be effective in growing normal and diseased field collected tissue; however, a procedure for controlling contamination must first be developed. The infection of normal callus tissue with sporidia of C. fusiforme and the culturing of naturally infected tissue offers an excellent opportunity for the study of the host-parasite phenomena under controlled conditions. The work of Harvey and Grasham (1969b 1969c and 1970) with C. ribicola lends impetus to corresponding work with C. fusiforme. The study of C. fusiforme in axenic culture may eventually lead to development of a control of this southern pine disease.

## SUMMARY

In order to better understand the nature of a particular plant disease, a thorough understanding of the life cycle of the causal agent is desirable. The ideal approach is to study the pathogen under controlled laboratory, greenhouse and field conditions. However, this is extremely difficult in the study of an obligate parasite since normal growth and development is dependent on the presence of living host cells. The use of plant tissue culture may alleviate this problem as indicated by the work of Harvey and Grasham (1967, 1969a, 1969b, 1969c and 1970) and Koenigs (1968) with C. ribicola.

In the present study, tissue cultures of loblolly pine, P. taeda, were used to develop callus on chemically defined culture media. The propagation of the obligate parasite, C. fusiforme, on callus would enhance further investigations of this organism.

In seven of the experimental tests, field collected diseased and normal tissue were utilized but none produced callus. This was due in part, at least, to contamination. The decontamination procedures of Harvey (1967) and Koenigs (1968) proved inadequate and that used by Walkinshaw et al, (1965) appeared to cause irreversible damage to the tissue. The use of 1/1000 $HgCl_2$ proved most effective for decontaminating seeds for tube grown seedlings and one month old seedlings grown under greenhouse conditions. However, this technique did not control contamination on field collected plant material.

Callus tissue was produced in those tests where tube and greenhouse grown seedlings were used. Callus tissue was light green in primary cultures and light greenish yellow in subcultures. When 0.1 mg 2,4-D was used, callus tissues were smooth textured whereas 1.0 and 2.0 mg 2,4-D produced rough textured callus tissues. It was demonstrated that callus was produced using Harvey's (1967) mineral salts with 20.0 g glucose, 6.0 g Bacto agar and 1.0 and 2.0 mg 2,4-D. As stated by Barnes and Naylor (1959), glycine inhibits growth of pine tissue. It was found that medium C containing glycine did not support growth. Subcultures proliferated only on medium C with glycine deleted. The addition of kinetin increased callus production but was not essential for callus initiation. Primary cultures turned brown after 30 days on medium containing kinetin while those tissues on medium lacking kinetin were not brown after 60 days. One subculture began to turn brown on the 30th day but when sectioned and implanted on fresh medium, proliferated and browning ceased.

These data indicate that stem sections of loblolly pine can be readily induced to form callus tissue in vitro and this tissue can be maintained for several months.

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